International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 12 No. 1, 2026
https://www.theaspd.com/ijes.php

Effect of Plant Growth Regulators on Physiology of Stevia rebaudiana Bertoni

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Abstract

Medicinal plants have long served as a major source for developing effective therapeutic agents. Excessive intake of sucrose-rich foods is closely linked with several metabolic disorders, including obesity, hypertension, atherosclerosis, coronary complications, high cholesterol levels, and sleep disturbances. Due to these risks, artificial sweeteners are frequently incorporated into beverages, confectionery items, sauces, pickles, and other food products. Stevia rebaudiana Bertoni offers a natural and safer alternative, particularly for individuals with diabetes, as its leaves contain highly sweet steviol glycosides. Secondary metabolites are influenced by plant growth regulators (PGRs) by varying the growth of plant and its physiological aspects. In the present study, Stevia plants treated with 25, 50 and 100 ppm of Indole-3-Acetic Acid (IAA) and Gibberellic Acid (GA_3) at 10 days interval for 60, 120 and 180 days. The findings revealed that GA_3 at 50ppm markedly enhanced protein and proline levels, whereas IAA at the same concentration significantly increased total carbohydrates and free amino acids compared with the control. Higher concentrations (100 ppm) of both PGRs showed a suppressive effect on all physiological parameters. Overall, the results suggest that low doses of IAA and GA_3 promote growth and improve physiological attributes of Stevia, with GA_3 being more effective for protein and proline enhancement and IAA for carbohydrate and amino acid accumulation.

KEYWORDS: *Stevia, plant growth regulators, GA_3, IAA, physiological responses.*

INTRODUCTION

Medicinal plants have been widely employed for treating numerous ailments since ancient period. From thousands of year, various plants are used in medicine and continue to this day (Grover et al., 2002; Chattopadhyay et al., 2007). Generations of traditional knowledge have resulted in the discovery of several treatments that have revolutionized healthcare. Local people all over the world have used medicinal herbs as a means of healing and curing diseases such as colds, infections and inflammation. Many pharmaceutical medications are derived from medicinal plants, which are important sources of bioactive chemicals that have health advantages. Analyses of herbal substances have been introduced, developed, and advanced at a rapid pace. These plants include compounds including alkaloids, flavanoids, and terpenoids that have antibacterial, antioxidant, and anticancer effects. As a result, these compounds are very useful in treating chronic conditions like diabetes and cardiovascular diseases. Their global relevance is enhanced by their natural and sustainable appeal.

India is naturally rich in diverse medicinal flora, that is why it is often described as a vast "herbal garden." Nearly three-quarters of the country's population resides in rural regions, where traditional plant-based knowledge remains a primary healthcare resource. In many tribal and Himalayan communities, people continue to rarely on locally available medicinal herbs, shrubs, and plant remedies to treat various ailments and infections. Rural people have inherited knowledge of traditional medicinal plant generation to generation. High-calorie foods and beverages, which are easily accessible, have contributed significantly to the rising number of health-related problems. Artificial sweeteners have adversely affected the human health (Grembecka, 2015). Due to this, the number of patients with obesity and diabetes are increasing, so that it is very necessary to educate the people for making healthy food choices.

Stevia rebaudiana Bertoni is a popular sugar substitute for managing diabetes and obesity. It is a natural perennial herb which is well-known for its high-intensity. "Candy leaf", "honey yerba","honey leaf" and meethi tulsi are its common name. It is non-caloric and nearly about300-fold sweeter compared to sucrose.

It is the member of asteraceae family and originated from South America (Singh & Rao, 2005; Reis et al., 2017). The height of mature plant reaches nearly about 65 cm to 180 cm in height if grown in the naturally fertile soil. The plant grows well in warm, sunny spot and sandy soil. It develops quickly like bushy shrub with branches position (Dwivedi, 1999). Some species of Stevia are *S. ani-sostemma, S. bertholdii, S. crenata, S.dianthoidea, S. enigmatica, S. lemmonii, S. micrantha, S. phlebophylla, S.rebaudiana, S. salicifolia,* and *S. viscid*. Carrera-Lanestosa et al., (2017) reported that stevia is a natural non- calorie sugar alternative and used for treating diabetes, high blood pressure, and obesity and their complications. Furthermore, it was demostrated that this herb may also safety consume by kids (Aguero et al., 2014). The glycoside which gives sweet taste to Stevia is identified by French Scientist Briedel and Lavieille in 1931. This glycoside is known as stevioside (Barriocanal et al., 2008). It has stevioside and rebaudioside - A, are two main active constituents. Its adaptability to a variety of environmental conditions and along with its expanding utilization in food and pharmaceutical industries highlight its significance in global health solutions. During various food and medicinal formulations, stevioside and rebaudioside- A constituents of Stevia maintain their stability across the conditions of temperature and pH (Abou-Arab AE et al., 2010). In India, stevia is planting on large scale because of its high demand as natural sweetener over the artificial sweeteners. It further exhibits health-promoting properties including antihypertensive, antimicrobial, and antioxidant effects. Growth hormones play important role in controlling and affecting physiological processes of the plants along with development, photosynthesis and assimilates accumulation. Auxin and gibberallic acids also alter the physiological properties of the plants (Darleen A.D. 2005). It has been reported that amino acid, carbohydrate as well as protein content was positively increased by GA_3 and IAA. IAA also increases total carbohydrate, free amino acids. When used in small concentrations they modify or regulate physiological processes in an appreciable measure in plants.

MATERIALS AND METHODS

One month old seedlings of *Stevia rebaudiana* Bertoni were purchased from CSIR, Institue of Himalyan Bioresource technology, Palampur, Himachal Pradesh, India. Experimental setup was carried out in green house and further test analysis was done in laboratory of Botany Department of Career Point University, Hamirpur (76°-17'50" to 76°-43'-42" eastern longitudes and 31°-24'-48" to 31°-53'-35" northern latitudes), Himachal Pradesh, India.

Experimental setup: *Stevia rebaudiana* Bertoni seedlings of forty five days old were transferred to the plastic pots of diameter 21 cm and height 19 cm in the month of June, 2023. Each pot contained 6 kg of a substrate composed of air-dried sandy soil, organic manure, and sand mixed in the proportion of 3:2:1.Plants were left for adaptation for nearly 15 days. Protection from rain was provided to the plants by using polythene cover. The polythene cover was pulled back immediately after the rain so that the plants receive maximum sunlight. To minimize positional influence on plant growth, the pots were rearranged every week. Subsequently, different solutions (25 mg/l, 50 mg/l and 100 mg/l) of both these growth regulators IAA and GA_3were exogenously applied separately to leaves of plants with the help of a hand sprayer. Each plant was sprayed with 20 ml of the various concentrations with the repeated applications of every ten days for 180 days. Twelve replicates were sprayed for each concentration. Plants of control groups were simply sprayed with distilled water. Samples used for study were collected at 60, 120 and 180 days of spraying and leaves from these samples were used to study different physiological and biochemical parameters.

Membrane stability index: Membrane stability index of leaf estimated by the method of Premchandra with modifications introduced by Sainnan (1994). In order to determine, membrane stability index of leaf, 2 mg fresh leaf of each treatment along with 20 ml of distilled water (distilled two times) was transferred to a Falcon tube. That falcon tube then kept inside the hot water bath at temperature of 40°C for about half an hour. Electrical conductivity (C_1) of this solution was recorded after heating with the help of conductivity meter. Thereafter, another set of leaf samples from the same plant kept inside another boiling water bath at 100°C for 10 minutes. Similarly, its conductivity (C_2) was also measured. Then, estimated membrane stability index of leaf samples by using the formula:

$$\text{MSI} = 1 - \frac{c1}{c2} \times 100$$

Carbohydrates: Carbohydrate content estimated in plant tissues using the method outlined by Hedge and Hofreiter (1962) through anthrone method. A 100 mg portion of the sample was placed in a test tube. Added 5 ml 2.5 N HCl to the sample test tube and then subjected to hydrolysis by keeping inside boiling water bath for 180 minutes. Then, allowed the sample to cool down at room temperature. The hydrolysate then neutralized using NA_2CO_3until all the effervencences ceased. The volume made to 100ml by adding distilled water and then processed this mixture by centrifugation at 10,000 rpm for 5 minutes. Then, took the aliquots of 0.5 and 1ml from the resulted supernatant for further evaluation. Standard solutions made by taking 0ml, 0.2ml, 0.4ml, 0.6ml, 0.8ml, and 1.0 mlin different test tubes from working standard solution. Test tube containing zero concentration served as the blank. In all test tubes, volume was adjusted to 1ml by adding distilled water. Thereafter, added 4ml anthrone reagent to each test tube. Then, placed all the test tubes inside the boiling water bath for eight minutes and cooled rapidly. Measured the resulting light green to dark green color at wavelength of 630nm. Prepared a calibration curve by plotting a graph between the concentrations of standard solution on x axis against their absorbance values on y axis. Using this graph, calculated the amount of carbohydrate presentin100 mg of sample.

$$\text{Total carbohydrate content present in 100 mg of the sample} = \frac{\text{glucose (mg)} \times 100}{\text{(Volume of test sample)}}$$

Total amino acids: Amino acids content was estimated by the following procedure given by Moore and Stein (1948). Approximately 500 mg of plant material weighed and ground it in pestle-mortar by adding a pinch of acid treated sand. The homogenized sample mixed with 5-10 ml 80% ethyl alcohol, centrifuged, and collected the supernatant. Residue extracted two times with 80% ethanol. All supernatants combined with each other. Pooled extract was concentrated by gentle evaporation when necessary. It used further for quantitative analysis of amino acids. For analysis, 0.1ml supernatant mixed with 1ml ninhydrin reagent and adjusted upto 2 ml by adding distilled water to it. Placed the mixture inside hot water bath for 20 minutes. After heating for 20 minutes, immediately added 5ml diluent and stirred thoroughly. After allowing solution to stand for 15 minutes, measured the absorbance of resulting purple color at 570nm by using reagent blank. Resulted color remained stable for up to one hour. The blank was prepared similarly, replacing the extract with 0.1 ml of 80% ethanol. Dissolved 50 mg leucine in 50 ml distilled water for preparing standard solution. Further diluted 10 ml stock solution to 100ml by adding distilled water for making working standard. Aliquots ranging from 0.1 to 1.0 mL provided a concentration range of 10-100 mg. These standards were processed in the same way as the samples, and recorded their absorbance values. Plotted a standard curve between concentration and absorbance. Then, calculated free amino acid content of the samples by this curve and expressed as leucine equivalents (%).

Protein: Protein content estimated by using the method given by Lowry et al. (1951). 500 mg of leaf sample in powder form with 5-10 percent of the phosphate buffer ground by using pestle and mortar. The supernatant obtained after centrifugation was taken for protein analysis. Transferred 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml, and 1 ml from working standard solution to separate test tubes. Pipetted 0.1 and 0.2 ml of supernatant to additional test tubes. Alltest tubes adjusted to 1 ml by adding distilled water. 1 ml distilled water in a test tube worked as blank. Added 5 ml reagent C (alkaline copper solution) to all test tubes along with test tube blank and stirred thoroughly. After proper mixing, left these test tubes undisturbed for ten minutes. Following this, 0.5 ml reagent D (Folin-Ciocalteu reagent) added to the solutions. Mixed well and incubate all the tubes in the dark for half an hour at room temperature. Blue color appeared in all test tubes. At last, recorded the absorbance of blue color solution of all test tubes at 660nm and prepared a calibration curve by using BSA. Then, calculated concentration of total protein present in the sample from this curve. The protein content expressed in mg/gm of sample as follows:

$$\text{Amount of protein content present in sample} = \frac{\text{mg of protein}}{\text{volume of test standard}} \text{conc. of standard}$$

Proline: Proline content analyzed by using reagent (acid ninhydrin) following procedure given by Bates et al., (1973). Homogenized a grounded leaf sample (200 mg) in liquid nitrogen with 4 ml 3% sulphosalicylic acid together. Centrifuged the homogenized mixture for 10 minutes at 3000 rpm. Mixed 2 ml filtrate, 2 ml acid ninhydrin solution along with 2ml glacial acetic acid in separate test tube. Then, incubate the test tube inside boiling water bath at 100°C for one hour and cooled rapidly between ice. Added 4 ml toluene to the all the test tubes after cooling. Then, vortexed vigorously for 15-20 seconds. There was formation

of two separate layers. Toluene layer separated carefully from these two layers and allowed it to reach upto the room temperature. By taking toluene as the blank, measured the absorption value another layer at 520 nm. Then, calculated the total proline content as:

$$\text{Amount of proline} = {}^{X}\times \frac{10}{2} \times \frac{1000}{250}$$

Amount of proline content present in leaf sample expressed in µg/g.

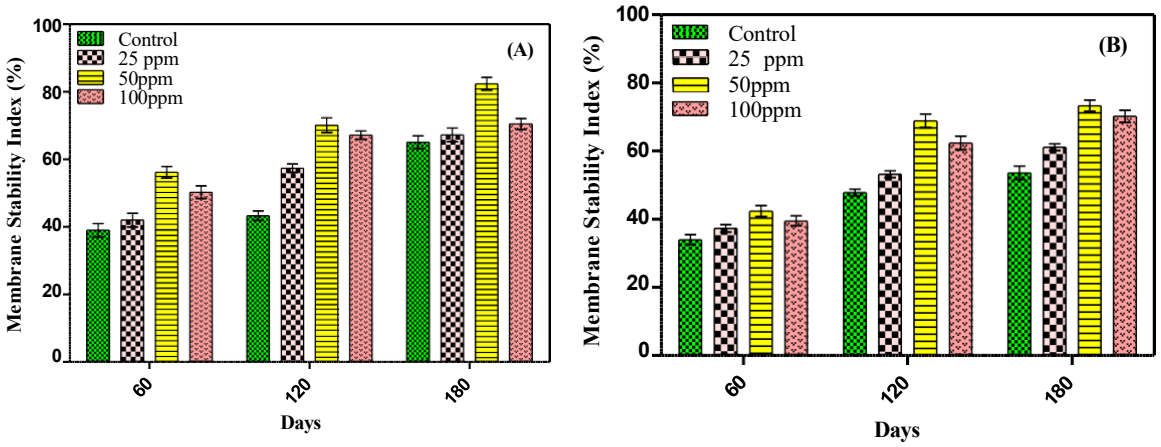
RESULTS AND DISCUSSION

Present study resulted that total amino acid and carbohydrate content was significantly increased after treatment with 50ppm of IAA and GA_3 respectively after 180 days of spraying as compared to control. On treatment with GA_3 50ppm protein and proline contents were maximum after 180 days of spraying than that of control. At 100 ppm of IAA and GA_3, all the physiological parameters were decreased after 60, 120 and 180 days of spraying as to 50 ppm of GA_3 and IAA.

Membrane Stability Index: Our experimental results show maximum membrane stability index at 50ppm of GA_3 after 180 days of spraying whereas the lowest membrane stability index valuehas been recorded in untreated plants after 60 days of spraying. Highest membrane stability index was noticed under 50ppm of GA_3 after 120 and 180 days of spraying whereas a decline in membrane stability index was noted in untreated plants 60 days post-spraying. Maximum increase in membrane stability index was exhibited by 50ppm GA_3 after 120 days of spraying which was about 38.231% in contrast to the control which whereas minimum increase (3.268%) in it was at observed at 25ppm of GA_3 after 180 days of spraying in response to control. The membrane stability index was highly reduced about (14.441%) at $GA_3$100ppm with respect to its 50ppm after 180 days of spraying (Table.1). From our results, it is cleared that stability of the cell membrane was more significantly increased at 50ppm of GA_3 after 60, 120 and 180 days, at GA_3 25ppm as well as at GA_3 100ppm after 120 days and ($P<0.001$)relative to the control plants. Non-significant increase in membrane stability index was observed at 25ppm of GA_3 after 60, 120 and 180 days as well as at 100ppm of GA_3 after 180 days of spraying ($P>0.05$). At 100ppm of GA_3 it was raised more significantly after 60 and 120 days of spraying and non significantly ($P>0.05$) increased after spraying for 180 significantly after days Fig. 1(A). Whereas, on treatment with 50ppm of IAA membrane

Table. 1 Membrane stability index of Stevia rebaudiana Bertoni under the impact of GA_3 and IAA with different concentrations. Mean ± SE (3 replicates); More significant (***); Significant (**); Less significant (*); Non-significant (NS).

Parameter	PGR	Treatments	60 days	120 days	180 days
Membrane Stability index (%)	GA3	Control	39.00±1.98	43.30±1.40	65.10±1.89
		25ppm	42.00^{NS}±2.01	57.40^{***}±1.23	67.30^{NS}±1.99
		50ppm	56.20^{***}±1.71	70.10^{***}±2.15	82.40^{***}±1.89
		100ppm	50.30^{***}±1.88	67.20^{***}±1.22	70.50^{NS} ±1.60
	IAA	Control	34.00±1.43	47.800±1	53.60±2
		25ppm	37.300^{NS} ±1.11	53.20^{NS} ±1	61.10^{***}±0.98
		50ppm	42.40^{**}±1.59	68.90^{***}±1.98	73.30^{***}±1.67
		100ppm	39.50±1.45	62.40±2	70.200±1.78



index was significantly increased 60 days after treatment (P<0.01) and more significantly increased after 120and 180 days of spraying when compared with control (P<0.001). It was non significantly increased under 25ppm of GA_3 after 60 and 180 days (P>0.05) but after 120 days of spraying it was more significantly increased after 120 and 180 days of spraying when compared with control (P<0.001). It increased non significantly (P>0.05) at IAA 25ppm after 60 and 120 days following spraying and more significantly increased in days after spraying (P<0.001) (Fig. 1B). At optimal concentrations, GA_3 and IAA positively enhanced cell membrane integrity by stimulating antioxidant enzyme activity (CAT, POD, SOD), which reduced lipid per-oxidation. These improve synthesis of structural proteins and phospholipids which strengthens membrane. GA_3 maintains membrane fluidity by promoting cell expansions and improving the water relations. The mechanism behind increasing the membrane stability index under the effect of GA_3 and IAA is that these regulates the membrane bound enzymes, stabilizes ion transport reduces electrolyte leakage which results in higher MSI. It also protects the membrane from the oxidative damage by enhanced proline and protein accumulation. Higher concentrations (100ppm) of both the PGRs causes hormonal imbalance which resulted a decrease in the membrane stability index of the Stevia plants. Excess amount of GA_3 and IAA increases reactive oxygen species which damages lipid contents of membrane and results in leakage. By this action, membrane stability index get reduced at higher doses of PGRs. Results obtained in the current investigation support the conclusions drawn by Miceli et al., (2019) which shows that the membrane stability index in *Lettuce* plant have been raised after spraying GA_3 exogenously to the leaves and also stimulate the plant growth**.** Also in the studies of Rady et al., (2021) *Vicia faba* plants have improved membrane stability index after treating exogenously with GA_3.

Amino acids: The highest amino acid content was also identified at 50ppm GA_3 after spraying duration of 180 days whereas least content of amino acid was detected under 50ppm GA_3 treatment and control after 60 days of spraying. Maximum level of amino acid was exhibited at 50ppm of GA_3 after 180 days of spraying which was about 33.374 % high as compared to control while minimum rise about 7.457 % in amino acid content have been obtained in at 25ppm of GA_3 after spraying for 120 days when compared with untreated plants. Minimum reduction about 2.301 % in amino acid extent was observed at a GA_3 concentration of 100 ppm while compared with 50ppm of GA_3after spraying for 60 days whereas it was highly reduced about 23.437 % at same concentration of GA_3 on comparing again with 50ppm of GA_3but after the duration of 180 days (Table 2). The amount Amino acid was increased more significantly (P<0.001) at 25ppm of GA_3after 180 spraying days and 50ppm $GA_3$for the period of 120 and 180 days when compared with control.Less significant increase was observed at 25 and 100 ppm of GA_3on comparing with control plants after 60 and 180 days after spraying respectively (P<0.05) whereas non significant increase in amino acid content was noticed at 25ppm of GA_3 after 120 days, 50ppm after 60 days and 100ppm within 60 and 120 spraying days in relation to control (P>0.05) Fig. 2(A). On

Table. 2 Amino acid content of Stevia rebaudiana Bertoni under the impact of GA_3 and IAA with different concentrations. Mean ± SE (3 replicates); More significant (***); Significant (**); Less significant (*); Non-significant (NS)

Parameter	PGR	Treatments	60 days	120 days	180 days
Amino acid (mg/g)	GA3	Control	37.45±2	47.90±1.98	48.07±1.54
		25ppm	43.48*±2.01	51.76^{NS}±1.34	60.24***±1.42
		50ppm	40.84^{NS} ±1.25	61***±0.99	72.15*** ±1.41
		100ppm	39.90^{NS} ±1.76	53.42^{NS} ±1.00	55.24*±2
	IAA	Control	36.56±2	40.93±1.98	49.56±1.54
		25ppm	43.09*±2.01	49.34**±1.34	62.13***±1.42
		50ppm	45.11**±1.25	57.90***±0.99	71.87***±1.41
		100ppm	39.78^{NS}±1.76	51.63***±1.00	69*** ±2

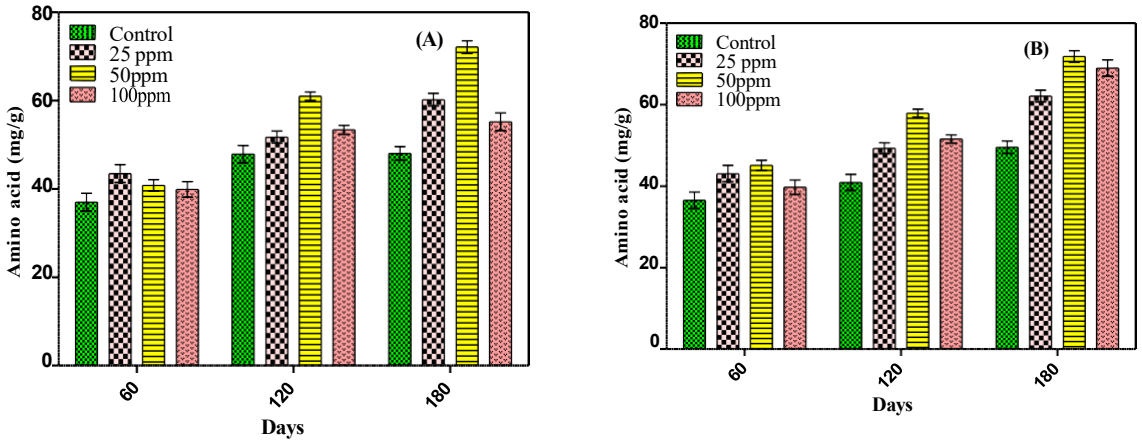


Fig. 2 Amino acid content of Stevia rebaudiana Bertoni under the impact of GA_3 (A) and IAA (B).

treatment with IAA 50ppm this content was increased significantly when treated for 60 days ($P<0.01$) whereas it was more significantly increased after 120 and 160 days in contrast to untreated plants ($P<0.001$). Non-significant increase was recorded on treating with 100ppm concentration of IAA after 60 spraying days as compared to plants that were not sprayed ($P>0.05$) and less significant increment was found on the treatment of IAA25ppm to plants for 60 spraying days as opposed to control group plants ($P<0.05$) Fig. 2(B). $GA_3$and IAA enhanced the amino acid content by stimulating nitrogen uptake and assimilation and by activating enzymes like reductase and glutamine synthetase. Enzymes activation enhanced proteins which in return released free amino acids. Under hormonal stimulation, amino acids act as osmoprotectants. Mechanically, GA_3 promoted the transcription and translation which increases the synthesis of amino acids among the plants and IAA improved the nutrient absorption by enhancing the root growth. This accumulation supports growth and metabolic activity. Maximum application of growth regulators reduces the enzyme activity and disturbs the nitrogen metabolism. Instead of synthesis of amino acid, energy oppositely enhances the stress responses. Findings of Hamida & Abdel (2013) are congruent to our results which reflected that the exogenous spray of GA_3 and IAA caused the positive enhancement in the amount of the amino acid content in *Triticum aestivum* plants. Also the investigation of Talat et al., (2020) shows that the amino acid content was promoted after the exogenously application of GA_3 to *Citrus aurantifolia* and *Citrus reticulate* plant.

Carbohydrate: In enhancing carbohydrate content in stevia plants IAA was found to be very effective in comparison to GA_3. Maximum amount of carbohydrate content 86.54mg was recorded at IAA 50 ppm for 180 spraying days whereas minimum carbohydrate content about 20 mg was noticed in control groups for 60 spraying days. Greatest rise in carbohydrate amount noticed at 50 ppm of GA_3 after 120 days of spraying

which was about 155.39 % more than that of control whereas small increase in it was seen at IAA 25ppm after 180 spraying days which was only 4.084% more than that of control plants. As like all the parameters carbohydrate content was also reduced at highest concentration 100ppm of both the plant growth regulators. The maximum reduction was reported at IAA 100ppm which was about14.351% less than that of IAA 50ppm for 180 days and the carbohydrate percentage was slightly reduced after spraying IAA 100ppm for 120 days that was 10.302% in comparison to 100ppm of GA_3 and IAA (Table. 3). Carbohydrate content was increased more significantly at$GA_3$50ppm when sprayed for 60, 120 and 180 days while compared with control plants ($P<0.001$) and significantly increased at 25ppm of $GA_3$for 60 days with respect to untreated plants ($P<0.01$). Non significant increase was observed at 25ppm and 100ppm of $GA_3$for 180 days on comparing with control ($P>0.05$) Fig. 3(A). However, for IAA, it was also increased more significantly on spraying with 50ppm of IAA for 60, 120 and 180 days when compared with control. Least significant increment in carbohydrate content was noticed on spraying 25ppm of IAA for 120 days in relation with control group plants ($P<0.05$). Similar to GA_3, on comparing with untreated plants non significant rise in carbohydrate content was seen at 25

Table.3: Carbohydrate content of Stevia rebaudianaBertoni under the impact of GA_3 and IAA with different concentrations. Mean ± SE (3 replicates);More significant (***); Significant (**); Less significant (*); Non significant (NS).

Parameter	PGR	Treatments	60 days	120 days	180 days
Carbohydrate content (mg/g)	GA3	Control	20.18±1.24	22.06±1.04	54.87±2
		25ppm	28.40**±1.76	43.45***±1.52	57.19^{NS}±1.11
		50ppm	39.38***±1.89	56.34***±1	69.02***±2.03
		100ppm	33.09***±1	47.50***±2.11	59.89^{NS} ±0.99
	IAA	Control	38.40±1.34	45.39±1.43	70.12±2.11
		25ppm	49***±1.21	52.03*±1	72.98^{NS} ±2
		50ppm	53***±1.62	59.11***±1.21	86.54***±2.04
		100ppm	46.3**±1	53.02**±2.11	74.12^{NS} ±1.22

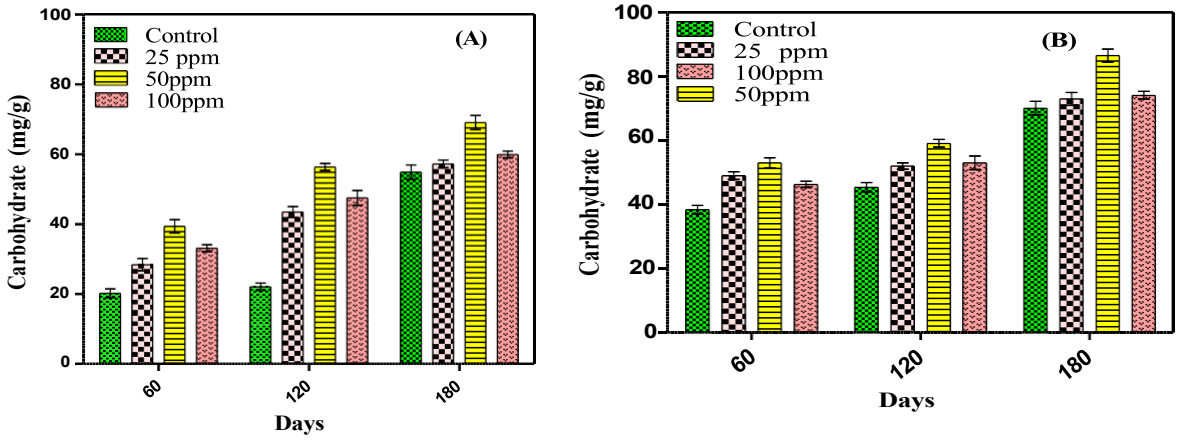


Fig. 3 Carbohydrate content of Stevia rebaudiana Bertoni under impact of GA_3 (A) and IAA (B).

and IAA 100ppm when sprayed for 180 days ($P>0.05$).Carbohydrate content was highly increased under IAA treatment because it enhances photosynthetic efficiency by increasing the chlorophyll content which

promotes photosynthate translocation. It improves the light interception by increasing the leaf expansion which leads to more accumulations of sugar in plants. IAA provides the mechanical support to the plant by stimulating cell division which enhances the photosynthate production. It increases the translocation of sugars from leaves to storage tissues. GA_3also raised the carbohydrate levels by promoting the activating enzymes in starch synthesis but less effectively than IAA. High level of growth hormones lowers the accumulation of carbohydrates by reducing source sink efficiency. Photosynthesis gets lowered due to the increasing metabolic stress. Kaur et al., (2000) noted the similar results for carbohydrate content which was enhanced after applying GA_3exogenously to the plant. Also increased the concentration of carbohydrate content in *Pisum sativum* plant under the effect of IAA whereas GA_3 did not show any significant influence in this plant (Khalid Hussain et al., 2020). IAA also increase the carbohydrate level in *Vicia faba* plant (Sadak et al., 2013).

Protein: In increasing protein content in stevia plant under the effect of IAA and GA_3, IAA have been showed the positive effect in comparison to GA_3. Greatest amount of protein content was observed at 50ppm of IAA after 180 days of spraying with respect untreated plants that was about while less protein content was seen in the untreated seedlings in response to treated seedlings while spraying for 60 days. Protein content was highly increased after applying IAA 50ppm for 180 days over control. This increase was about 96.488% more than that of control. However, smallest rise about 16.375% was noticed on spraying IAA 25ppm for 60 days on comparing with control. A small reduction was noticed at 100ppm of GA_3 after 60 days of with respect to $GA_3$50ppm which was only 15.334% less than that of whereas maximum reduction 31.451% was noticed at 100ppm of IAA with respect to 50ppm of IAA after 120 days (Table. 4). More significant increase in protein

Table. 4 Protein content of Stevia rebaudiana Bertoni under the impact of GA3 and IAA with different concentrations. Mean ± SE (3 replicates); More significant (***); Significant (**); Less significant (*); Non-significant (NS).

Parameter	PGR	Treatments	60 days	120 days	180 days
Protein (mg/g)	GA3	Control	15.03±1.84	23±2.87	34.98±1.99
		25ppm	19.11^{NS}±2	33.65**±1.84	43*±1.12
		50ppm	24.78**±1.95	39.60***±2	51.23***±1.81
		100ppm	20.98 ^{NS} ±1.31	30 ^{NS} ±2.06	49.87***±2.29
	IAA	Control	20.03±1.54	29±2.87	32.18±1.99
		25ppm	23.31 ^{NS} ±2	39.65**±1.84	45***±1.12
		50ppm	35.7***±1.06	49.60***±2	63.23***±1.81
		100ppm	25.08 ^{NS} ±1.31	34 ^{NS}±2.06	50.87***±2.29

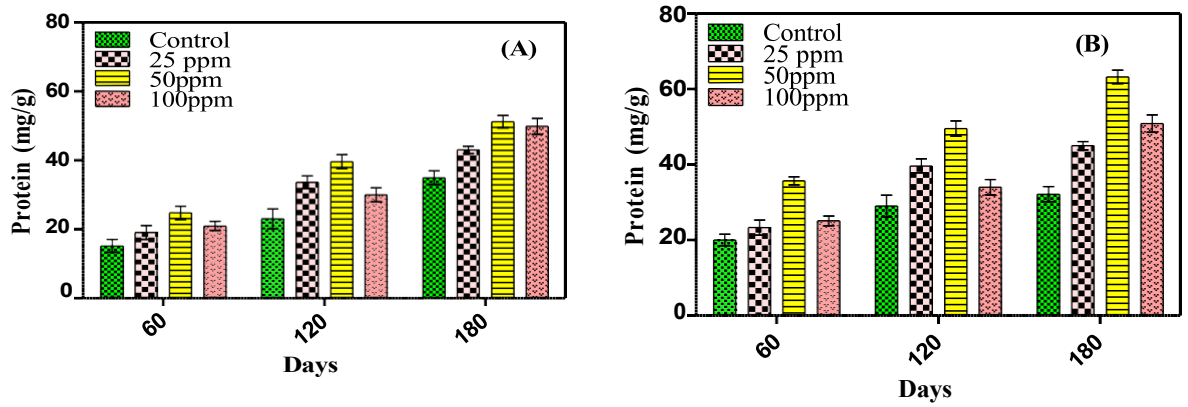


Fig. 4 Protein content of Stevia rebaudiana Bertoni under impact of GA_3 (A) and IAA (B).

amount was observed on applying 50ppm GA_3 for 120 and 180 days and also at 100ppm of GA_3 for 180 days ($P<0.001$).On spraying 25ppm $GA_3$for 120 days and 50ppm of GA_3 for 60 days, we seen a significant increase with respect to untreated plants ($P<0.01$). Protein content was less significantly raised on treatment with GA_3 25ppm for 180 days ($P<0.05$). Non-significant increase was examined in protein level after spraying with 25ppm of GA_3 for 60 days and 100 ppm of GA_3 for 60 and 120 days in response to untreated plants ($P>0.05$) Fig. 4(A). Whereas, we observed more significant increase in case of IAA in enhancing protein concentration on treatment with 50ppm of IAA for 60, 120 and 180 days and with 25ppm and 100ppm of IAA for 180 days of spraying on comparing with control ($P<0.001$). It was significantly increased with the application of IAA 25ppm for 120 days against control ($P<0.01$) whereas non significant increase was noticed at the application of IAA 25ppm for 60 days and IAA100ppm for 60 and 120 days on contrasting with control grouped plants ($P>0.05$) Fig. 4(B). Protein synthesis is strongly promoted by IAA. It also enhances the RNA synthesis and ribosomal activity. Protein level is enhanced by improving nitrogen assimilation and amino acid utilization. Gene expression which is responsible for the protein synthesis activated by IAA. GA_3 also supports protein accumulation by stimulating the enzyme production but less comparatively to the IAA. However, higher concentration of plant growth regulators causes stress which leads to the degradation of protein in the plants. It also inhibits the functioning of ribosomes and increased the protease activity which results in the reduction of net protein content. Our outcomes were also familiar with the findings of Muthulakshmi and Pandiyarajan, (2013) also stated that IAA application enhanced the protein synthesis in *Catharanthus roseus*. Khalid et al., (2016) reported in their studies that after applying 50mg L^{-1} of GA_3 exogenously protein content was raised up to the maximum level seeds of three medicinal plants *Coriander sativum, Mentha arvenses and Ocimum sativum.* However, Alrashdi et al., (2017) stated in their findings that the exogenous foliar treatment of GA_3 at different concentration caused a significant response in uplifting the protein level.

Table. 5 Proline content of Stevia rebaudiana Bertoni under the impact of GA_3 and IAA with different concentrations. Mean ± SE(3 replicates); More significant (***); Significant (**); Less significant (*); Non significant (NS).

Parameter	PGR	Treatments	60 days	120 days	180 days
Proline (mg/g)	GA3	Control	9.5±0.84	13.2±1	16±0.12
		25ppm	11.2^{NS}±0.88	21***±0.63	26.1***±1.23
		50ppm	17.30***±1.11	26.60***±1.0	33.80***±0.92
		100ppm	10.50^{NS} ±1.10	17.80*±1.66	29***±0.78
		Control	10±0.99	11.09±1.30	15.90±1.20

	IAA	25ppm	11.09 ^{NS} ±0.73	13.90 ^{NS} ±0.98	21.34**±1.64
		50ppm	15.76**±0.77	25.09***±1.00	29.12***±0.99
		100ppm	11 ^{NS} ±1	19.45***±1.56	21.09*±1.52

Proline: Proline content was positively enhanced in stevia plants under the effect of GA_3. After applying 50ppm of GA_3 for a period of 180 days, we obtained the highest concentration of proline content whereas, minimum amount of proline was gained at 25ppm of GA_3 after 60 days of spraying. In proline content, maximum increase was observed at 25 ppm of GA_3 after 180 days which was about 63.125% more than that of control plants while it was slightly increased on treatment with 25ppm of IAA for 60 days of spraying which was 10.9% less than that of control. Proline content reduced at high concentration of these two PGR's. It was highly reduced at 100ppm of GA_3 with respect to 50ppm of GA_3 after 120 days which was about 33.082% less than that of control while less decreased at IAA 100ppm for 60 days whereas it was only 30.203% less than that of 50ppm of IAA and GA_3 (Table. 5). Non significant increase in proline content was found at 25ppm and 100ppm of GA_3 60 days of spraying on comparing with control plants ($P>0.05$) and less significant rise in it recorded under treatment with 100ppm of GA_3 for 120 days of spraying as compared to control ($P<0.05$).However, it increased more significantly on treating the seedling with 50ppm of GA_3 after 60, 120 and 180 days of spraying, on treating with 25ppmof GA_3 for 120 and 180 days of spraying and also on spraying with 100ppm of GA_3 for

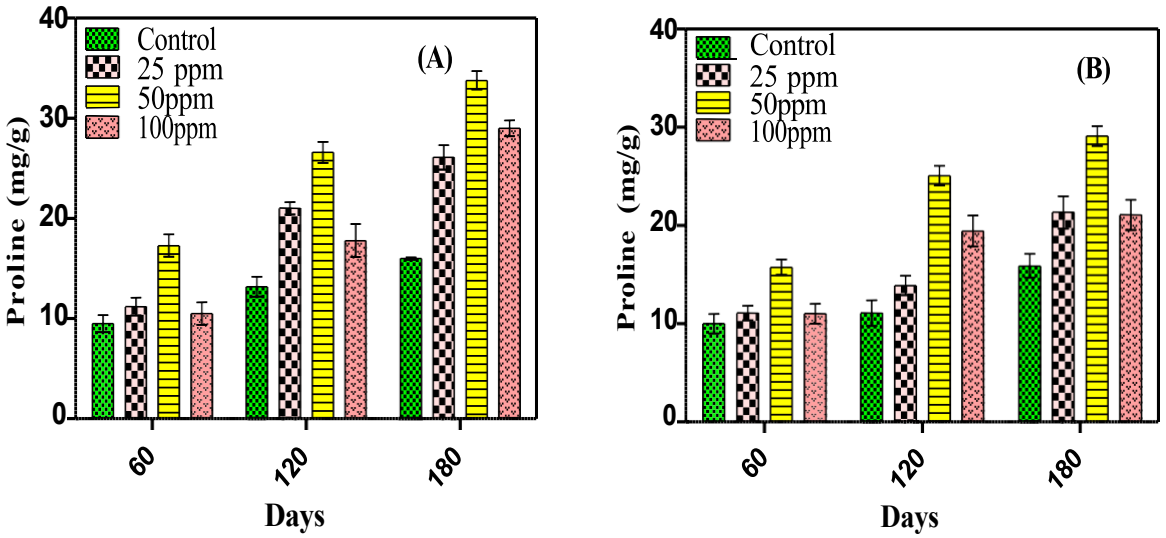


Fig. 5 Proline content of Stevia rebaudiana Bertoni under impact of GA_3 (A) and IAA (B)

180 days ($P<0.001$) Fig. 5(A).In case of IAA, the proline content was increased non-significantly on applying 25ppm of IAA for 60 and 120 days and also after applying exogenously 100ppm of IAA for 60days while comparing with control plants. Less significant increase was noticed on applying IAA 100ppm exogenously to the leaves for 180 days ($P<0.05$). It was significantly increased on treating with 25ppm and 50ppm of IAA after 180 and 60 days respectively in relation to control ($P<0.01$). Proline content was more significantly increased after the application of IAA 50ppm for120 and 180 days and also on treatment with IAA 100ppm for 120 days while comparing with control plants ($P<0.001$). Proline content was significantly increased under the effect of GA_3 in comparison to IAA. GA_3 induces moderate physiological stress which triggers proline synthesis. GA_3 increases proline biosynthesis by activating enzymes of glutamatic pathway. Free redicals are scavenged by proline which maintains osmatic balance. It also helps in maintaining membrane stability and cellular hydration. Excess amount of GA_3 and IAA causes metabolic toxicity and pathways of proline synthesis are inhibited which results in the distruption of amino acid metabolism. Our observations were also congruent to the findings of Slabert et al., (2014) showing an accumulation of proline content in *Amaranthus* plants by

the exogenous foliar application of GA_3. Again, Munteanu et al., (2014) concluded from their studies that after applying GA_3 to the leaves of *Brassica napus* L. proline content was highly raised upto its maximum level. Multiple studies revealed the positive effects plant growth regulators on the physiological parameters of numerous medicinal plants. Ruinowska et al., 2025 reported from their findings that there was a positive influence in enhancing the relative water content in *Polygonatum multiflorum* (L.) after treatment with GA_3 400 mg L^{-1}.Promotory effect of GA_3 have been exposed in improving the physiology of the plant by enhancing protein content and memebrane stability index (17.34 %, 13.76 %, and 18.58 %) in *Gladiolus grandiflorus* Gaur et al., (2022). In Euglena, Hakim et al., (2023) reported that the carbohydrate content was positively enhanced on applying IAA 15g/L. The percentage of the amino acid content was also increased when the seeds of *Salvia hispanica* L. were treated with 0.14 g/L of GA_3 Costa et al., (2021). However, it was reported by Yu et al., (2012) that after the exogenous application of GA_3 total free amino acid content was decreased in *Paris polyphylla.* Aldesuquy et al., (2018) noticed that the protein content was increased in *Pisum sativum* under the treatment IAA. Again protein level was uplifted in *Sesbania pea* through the application of GA_3 Krishna and Mahadevaswamy, (2019). Similar outcomes were reported by Khalid and Aftab (2020) in *Solanum tuberosum* under the treatment with both IAA and GA_3.Liu et al., (2025) also observed the positive influence of GA_3 in rising the protein content (18.65%) in *American ginseng* when sprayed for 90 days. Application of 0.1, 0.2 and 0.3 g/L of GA_3 to *Oryza sativa* L resulted the significant increase in the protein content Guo et al., (2022). Lamlom et al., (2025) also reported the positive impact of 0.1 g/L and 0.2 g/L GA_3in the maximizing the concentration of proline content of *Triticum aestivum* L.

CONCLUSION:

From the above study, we concluded that after applying the various concentration of both the PGR's the physiological parameters of *Stevia rebaudiana* plants have been promoted. Among both the plant growth regulators, GA_3 was found to very effective in enhancing the physiology of the plant. As, GA_3 positively upgraded the membrane stability index, amino acid and proline content whereas IAA had showed the positive effect in enhancing the carbohydrate and protein content. At higher concentration (100ppm) of both the PGR's resulted in the reduction of all the physiological parameters of the plant. Although, at all the concentrations (25ppm and 50ppm), these parameters were increased significantly. All the physiological parameters were enhanced at lower concentrations of GA_3 and IAA because of regulation of enzyme, membrane integrity and improvement in metabolic activity whereas all the parameters were declined at higher concentration due to oxidative stress, hormonal toxicity and metabolic imbalance among plants.GA_3 and IAA act as growth promoters only at low concentrations (25 and 50ppm) and enhance enzyme activity, photosynthesis, nutrient uptake and cellular metabolism whereas higher concentration (100ppm) causes hormonal imbalance, metabolic disruption, oxidative stress, reduced synthesis and enhanced degradation of bio-molecules in plants. By the use of these two growth regulators (IAA and GA_3), Secondary metabolites of the plants can be increased in large extent which results in enhancing the therapeutic properties of plant.

Acknowledgement

The authors are grateful to the Career Point University, Hamirpur, Himachal Pradesh, India for providing facilities and encouragement for the research.

Funding

No funding was received for conducting this study.

Data Availability

Not Applicable

Declarations

Ethical Approval

Nor applicable

Competing Interests

The authors declare that they have no conflict of interest.

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